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Indian Standard

METHOD FOR ESTIMATION OF AMINO ACIDS IN FOOD

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Indian Standard

METHOD FOR ESTIMATION OF AMINO ACIDS IN FOOD

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Indian Standard

METHOD FOR ESTIMATION OF AMINO ACIDS IN FOOD

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 21 October 1975, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Amino acids are evaluated in a large number of proteinous foods. There are mainly two methods, namely, chemical and microbiological for amino acid analysis. Of these two methods, the microbiological method is simpler, less cumbersome and offers reproducible results, especially when amino acid analyser is not available in most of the laboratories owing to its high cost. However, with a view to avoiding interlaboratory differences in microbiological method which may affect the ultimate results, this standard has been formulated. It is expected that this method will help in achieving uniformity in methods of analysis of amino acids thereby facilitating interpretation and comparison of results.

0.3 In reporting the results of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

1. SCOPE

1.1 This standard prescribes a microbiological method for estimation of essential amino acids in food.

2. PRINCIPLE

2.1 The method is based on the observation that certain micro-organisms require specific nutrients for growth. Using a basal medium complete in all respects except for the amino acid under test, growth responses of the organisms are compared quantitatively in standard and in unknown solutions. Either the acid or the turbidity produced by the organisms is measured to determine the extent of growth and thereby the amount of nutrient in the test solution.

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*Rules for rounding off numerical values (*revised*).

3. QUALITY OF REAGENTS

3.1 Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1960*) shall be employed in the test.

NOTE — ' Pure chemicals ' shall mean chemicals that do not contain impurities which affect the test results.

4. MICROBIOLOGICAL ASSAY OF AMINO ACIDS

4.1 Test Organisms

4.1.1 *Leuconostoc Mesenteroides* (*ATCC No. 8042*) — For use in assay of all amino acids except threonine.

4.1.2 *Streptococcus Faecalis* (*ATCC No. 9790*) — For use in assay of threonine.

NOTE — *Lactobacillus plantarum* (*ATCC No. 8014*) may also be used in assay of isoleucine, leucine, methionine, phenylalanine, tryptophan and valine.

4.2 Preparation and Maintenance of Stock Culture — Prepare stock culture of agar tubes and inoculate with the pure culture (appropriate organisms to be used for the different assays). Incubate the tubes for 16 to 24 hours at 37°C and store at 4°C. Transfer the cultures into new agar tubes every fortnight.

4.3 Preparation of Stock Culture Tubes

4.3.1 *Salt Solution A* — Dissolve 25 g each of dibasic potassium phosphate (K_2HPO_4) and monobasic potassium phosphate (KH_2PO_4) in water and make up to 250 ml with water.

4.3.2 *Salt Solution B* — Dissolve the following salts in 250 ml of water to which are added a few drops of concentrated hydrochloric acid to obtain a clear solution. Store under toluene.

Magnesium sulphate ($MgSO_4 . 7H_2O$)	10·0 g
Sodium chloride (NaCl)	0·5 g
Ferric sulphate ($FeSO_4 . 7H_2O$)	0·5 g
Manganese sulphate ($MnSO_4 . H_2O$)	0·5 g

4.3.3 Dissolve the following ingredients in 200 ml of distilled water, adjust the *p*H to 6·8 and make the volume up to 250 ml. Separately dissolve 7·5 g of agar (*see* IS : 6850-1973†) in 250 ml of distilled water by heating. Mix well both the solutions together. While the solution is still hot, take 10 ml of the solution into test tubes. Plug with cotton, and autoclave

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*Specification for water, distilled quality (*revised*).

†Specification for agar, microbiological grade.

for 15 minutes at 82 KN/m^2 (0·84 kgf/cm^2) pressure. After cooling to room temperature store the tubes at 2 to 4°C and use for maintaining stock cultures.

Peptone (*see* IS : 6853-1973*)	5·0 g
Yeast extract (*see* IS : 7003-1973†)	1 0 g
Glucose, anhydrous	10·0 g
Sodium acetate $3H_2O$	17·0 g
or	
Sodium acetate, anhydrous	10·0 g
Salt Solution A	2·5 ml
Salt Solution B	2·5 ml

4.4 Preparation of Inoculum Broth — Prepare the inoculum broth in the same way as the culture medium (*see* **4.3**) excepting, that instead of the agar solution, use more of distilled water (after adjusting the *p*H to 6·8) to make up the volume to 500 ml. Take 10 ml of the solution into test tubes, then plug with cotton, autoclave for 15 minutes at 82 KN/m^2 (0·84 kgf/cm^2) pressure, cool to room temperature and store at 2 to 4°C.

4.5 Preparation of Inoculum — A day prior to use, inoculate inoculum broth tube with a loopful of culture from the stock culture and incubate at 37°C for 16 to 18 hours. Use a centrifuge tube plugged with cotton, an all glass syringe, a 0·8-mm needle, and some saline (0·9 percent) in a conical flask. Sterilize saline by autoclaving for 15 minutes at 103 KN/m^2 (1·05 kgf/cm^2) pressure and syringe, needle and centrifuge tube by hot air-oven at 160°C for one hour. Transfer the cells from the inoculum tube to the centrifuge tube and centrifuge, decant off the supernatant and re-suspend the cells in sterile saline and centrifuge. Repeat the process two or three times. Again make up the washed cells to a suspension using the saline. Take re-suspended cells in the sterile syringe and inoculate the assay tubes with one drop each of the inoculum. The optical density of the inoculum should be about 0·1.

5. PREPARATION OF SOLUTIONS FOR ASSAY TUBES

5.1 Salt Solution A — Dissolve 12 g each of dibasic potassium phosphate (K_2HPO_4) and monobasic potassium phosphate (KH_2PO_4) in water and make up to 100 ml with water.

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*Specification for peptone, microbiological grade.

†Specification for yeast extract, microbiological grade.

5.2 Salt Solution B — Mix and dissolve the following in water to make *100 ml:*

Magnesium sulphate ($MgSO_4 . 7H_2O$)	4·0 g
Manganese sulphate ($MnSO_4 . 4H_2O$)	0·4 g
Ferric sulphate ($FeSO_4 . 7H_2O$)	0·2 g
Sodium chloride (NaCl)	0·2 g

5.3 Adenine-Guanine-Uracil (AGU) Solution — Dissolve 0·2 g of each of adenine sulphate, guanine hydrochloric acid and uracil in 100 ml of water using concentrated hydrochloric acid drop-wise for dissolving.

5.4 Xanthine Solution — Dissolve 0·2 g of xanthine in water using liquor ammonia drop-wise for dissolving and make up to 100 ml with water.

5.5 Vitamin Solution

Para-amino benzoic acid (PABA)	2·00 g
Biotin	0·02 mg
Ca-panthothenate	10·00 mg
Folic acid	0·20 mg
Niacin	20·00 mg
Pyridoxal HCl	6·00 mg
Pyridoxamine 2 HCl	20·00 mg
Pyridoxine HCl	10·00 mg
Riboflavin (*see* Note)	10·00 mg
Thiamine	10·00 mg

Dissolve in water and make up to 100 ml.

NOTE — Riboflavin is weighed separately, dissolved in a few millilitres of acetic acid and mixed with the other solution.

5.6 Non-essential Amino Acid Solution

dl-alanine	4·0 g
l-asparagine	8·0 g
l-aspartic acid	2·0 g
l-cystine	1·0 g
l-glutamic acid	6·0 g
Glycine	2·0 g
l-proline	2·0 g
dl-serine	1·0 g
l-tyrosine	2·0 g

Dissolve these amino acids in water and make up to 100 ml using concentrated hydrochloric acid dropwise for dissolving.

5.7 Essential Amino Acids

	g/100 ml
l-arginine HCl	4·85
l-histidine HCl	1·24
dl-Isoleucine	5·00
l-leucine	2·50
l-lysine HCl	5·00
dl-phenylalanine	2·00
dl-threonine	4·00
dl-tryptophan	0·80
dl-valine	5·00
dl-methionine	2·00

Dissolve each of these amino acids in water and make up to 100 ml using concentrated hydrochloric acid drop-wise for dissolving, and keep each amino acid solution in separate bottles.

5.8 Composition of Basal Medium

5.8.1 *For Leuconostoc Mesenteroides*

Glucose	5·0 g
Sodium acetate	4·0 g
Ammonium chloride	0·6 g
Salt solution A	1·0 ml
Salt solution B	1·0 ml
AGU solution	1·0 ml
Xanthine solution	1·0 ml
Vitamin solution	1·0 ml
Non-essential amino acid solution	1·0 ml
l-arginine HCl	1·0 ml
l-histidine HCl	1·0 ml
dl-isoleucine	1·0 ml
l-lysine HCl	1·0 ml
dl-phenylalanine	1·0 ml
dl-threonine	1·0 ml
dl-tryptophan	1·0 ml
dl-valine	1·0 ml
dl-methionine	1·0 ml
l-leucine	1·0 ml

Mix well the solution, adjust the *p*H to 6·8 and make the volume up to 100 ml with water.

5.8.2 *For Streptococcus Faecalis*

Hydrogen peroxide (H_2O_2) treated peptone (*see* IS : 6853-1973*)	75 ml
l-methionine	100 mg
l-cystine	100 mg
l-tyrosine	100 mg
dl-tryptophan	200 mg
Glycine	100 mg
Glucose (anhydrous)	20 g
Sodium acetate (hydrated)	33 g
Ammonium chloride	6 g
AGU solution	12 ml
Xanthine solution	12 ml
Salt solution A	5 ml
Salt solution B	5 ml

Vitamin Additions

Aneurine	1 000 µg
Calcium pantothenate	1 000 µg
Nicotinic acid	2 000 µg
Riboflavin	2 000 µg
Pyridoxine HCl	1 600 µg
p-amino benzoic acid (PABA)	50 µg
Water to make	500 ml

Mix the solution well, adjust the *p*H to 6·8.

NOTE — In the preparation of the basal medium, the amino acid to be assayed is omitted.

5.9 Preparation of Stock Standard Solution — Use for the preparation of standard solutions *l*-form of the amino acids. Dissolve 50 mg of the amino acid in 100 ml of water to make 500 µg/ml. A few drops of concentrated hydrochloric acid may be needed to dissolve some of the amino acids. The standard stock solution should be prepared fresh every three months.

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*Specification for peptone, microbiological grade.

5.10 Working Standard Range

	Range (µg)	*Dilution* (ml)
Arginine	0 to 40	4 to 50
Histidine	0 „ 10	2 „ 100
Isoleucine	0 „ 25	2·5 „ 50
Leucine	0 „ 25	5 „ 100
Valine	0 „ 25	1 „ 50
Methionine	0 „ 10	1 „ 50
Phenylalanine	0 „ 10	2 „ 50
Threonine	0 „ 20	1 „ 100
Tryptophan	0 „ 10	1 „ 50
Lysine	0 „ 30	3 „ 50
Cystine	0 „ 5	1 „ 100
Tyrosine	0 „ 10	2 „ 100

NOTE — Make the dilutions from a stock standard solution containing 500 µg amino acid per ml.

5.11 Standard Levels — Take 0, 0·1, 0·2, 0·3, 0·4, 0·5, 0·6, 0·7, 0·8 and 1·0 ml in triplicate, add enough water to make 1 ml and follow by 1 ml of basal medium. Cover the tubes with cotton wool plugs and wrap with brown (kraft) paper to protect the cotton wool plugs from getting wet during autoclaving. Alternatively use aluminium caps.

5.12 Preparation of Sample

5.12.1 *Acid Hydrolysis* — Add 1 g of the sample to 25 ml of 2·5 N hydrochloric acid. Autoclave the mixture for 6 hours at 103 kN/m^2 (1·05 kgf/cm^2) pressure. Cool and add 2 ml of 2·5 ml sodium acetate. Adjust the *p*H to 4·5 and make the solution up to a known volume and filter. Take an aliquot and adjust the *p*H to 6·8 with sodium hydroxide, and dilute to the required concentration.

5.12.2 *Alkali Hydrolysis for Estimation of Tryptophan and Tyrosine Only* — Add 1 g of the sample to 25 ml of 2 N sodium hydroxide and autoclave the mixture for 6 hours or add 5 g of barium oxide to 25 ml of water and autoclave for 8 hours at 103 kN/m^2 (1·05 kgf/cm^2) pressure. Then cool to room temperature, adjust the *p*H to 4·0 with either hydrochloric acid or acetic acid and make up to a known volume and filter. Adjust *p*H of the aliquot at 6·8 and dilute to get the required concentration. Recemisation takes place during this treatment and the amino acids will all be in *dl*-form.

5.12.3 Sample should be preserved and stored under toluene at 4°C and used for analysis preferably within a month's time.

5.13 Sample Levels — Take 0·2, 0·4, 0·6, 0·8 and 1·0 ml in duplicate, add enough water to make to 1 ml and follow by 1 ml of the basal medium. Cover the tubes with cotton wool plugs and wrap with brown (kraft) paper to protect the cotton wool plug from getting wet during autoclaving. Alternatively use aluminium caps.

6. PROCEDURE

6.1 Standard Tubes — Sterilize the tubes of basal medium containing standard levels of amino acid as in **5.11** by autoclaving at 120°C for 10 minutes. Cool to room temperature and inoculate 3 tubes of each standard with the inoculum as in **4.5**. For acidimetric titration method (*see* **6.3**), incubate the inoculated tubes at 37°C for 72 hours, except for cystine where it should be done for 18 to 24 hours. If turbidimetric method (*see* **6.4**) is used, incubate the inoculated tubes at 37°C for 16 to 20 hours.

6.2 Sample Tubes — Sterilize the tubes of samples (**5.13**) as in **6.1**.

6.3 Acidimetric Method

6.3.1 *Standard Solution* — 0·02 N sodium hydroxide and 0·1 percent bromothymol blue indicator solution.

6.3.2 Transfer the contents quantitatively to a 150-ml conical flask, rinsing the tubes with distilled water twice. Add about 0·2 ml of 0·1 percent bromothymol blue indicator and titrate the solution against 0·02 H sodium hydroxide to an end point of greenish blue colour around *p*H 6·8. A *p*H meter may be used in place of the indicator solution.

6.3.3 *Calculation* — Draw a standard curve for the assay by plotting the volume of 0·02 H sodium hydroxide on the *Y*-axis against concentration of the amino acid per tube in the standard series on the *X*-axis. It is preferable to plot these on logarithmic scale in order to obtain a straight line or a type of curve where the straight line of the curve can be utilized to determine amino acid content in the sample tubes. Determine amino acid content of tubes in the unknown series by interpolation of the titre values on the standard curve. Calculate the average for 1 ml of test solution from values obtained from not less than three tubes which do not vary by more than 10 percent of the average. Calculate the amino acid content of the test solution using the following relationship:

$$\mu\text{g of test amino acid/g sample} = \frac{\text{Average } \mu\text{g per ml} \times \text{volume} \times \text{diluting factor}}{\text{Mass of the sample}}$$

6.4 Turbidimetric Method

6.4.1 *Apparatus* — Nephalometer (turbidimeter).

6.4.2 *Method* — Take nephalometric readings of the growths using the tube supplied with the nephalometer. Transfer the same known volumes of growths in the standard tubes, commencing with the lowest amount of amino acid, that is, the highest dilution made. Draw a standard curve for the assay by plotting the turbidimetric readings on the *Y*-axis against concentration of the amino acid per tube in the standard series on the *X*-axis. It is preferable to plot these on a logarithmic scale in order to obtain a straight line or a type of curve where the straight line of the curve can be utilized to determine the amino acid content in the sample tubes. Determine the amino acid content of the sample tubes by interpolation of the turbidimetric values on the standard curve. Calculate the average for 1 ml of test solution from values obtained for not less than three tubes which do not vary by more than 10 percent of the average. Calculate the amino acid content of the test solution as in 6.3.3.

NOTE — It is essential that the standard curves as in 6.3.3 and 6.4.2 be constructed each time that an assay is undertaken since conditions of autoclaving, temperature of incubation, etc, which influence the standard curve readings, cannot be duplicated exactly from time to time.

7. REPEATABILITY

7.1 The method should be repeatable within the range of ± 10 percent.

INTERNATIONAL SYSTEM OF UNITS (SI UNITS)

Base Units

QUANTITY	UNIT	SYMBOL
Length	metre	m
Mass	kilogram	kg
Time	second	s
Electric current	ampere	A
Thermodynamic temperature	kelvin	K
Luminous intensity	candela	cd
Amount of substance	mole	mol

Supplementary Units

QUANTITY	UNIT	SYMBOL
Plane angle	radian	rad
Solid angle	steradian	sr

Derived Units

QUANTITY	UNIT	SYMBOL	DEFINITION
Force	newton	N	1 N = 1 kg.m/s^2
Energy	joule	J	1 J = 1 N.m
Power	watt	W	1 W = 1 J/s
Flux	weber	Wb	1 Wb = 1 V.s
Flux density	tesla	T	1 T = 1 Wb/m^2
Frequency	hertz	Hz	1 Hz = 1 c/s (s^{-1})
Electric conductance	siemens	S	1 S = 1 A/V
Electromotive force	volt	V	1 V = 1 W/A
Pressure, stress	pascal	Pa	1 Pa = 1 N/m^2

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